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Proteolytic enzyme production by *Fusarium oxysporium* under solid-state fermentation.

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ABSTRACT:
The production of protease by *Fusarium oxysporium* Schlecht. was studied under solid-state fermentation. *F. oxysporium* isolated from infected soybean seeds produced 1.19U of extra cellular protease/g of solid substrate in 8 days of incubation period. Enzyme production in submerged fermentation was found to be less than solid-state fermentation. The effects of fermentation condition such as substrate concentration, pH of the medium, temperature of incubation and inoculum concentration for enzyme production were investigated
Keywords: Solid-state fermentation, *Fusarium oxysporum*, Soybean meal

INTRODUCTION

Microbial proteases has almost replaced proteolytic enzyme of animal and plant origin. The fermentation industry has developed methods for low cost production of proteolytic enzyme of high purity from microbial source. Annual sale of Proteolytic enzymes is estimated to be over U.S. $100 million. About 75% of total enzyme is used in detergent industry and 10% in Dairy industry and rest 15% in number of minor application in pharmaceutical, food processing, meat tenderization, tanning, brewing, baking, animal feed, textile and other industries

Solid-state fermentation (SSF) is preferred to submerged fermentation (SmF) due to its numerous advantages [1]. Recently Pandey *et al* [2] reviewed production of proteolytic enzymes in SSF systems. Species of *Aspergillus*, *Penicillium*, *Rhizopus* and *Mucor* are known to produce variety of proteases. Saxena and Prasad [3] have emphasized the secretion of protease by *Fusarium solani* in the pathogenesis of Tomota- *Fusarium solani* interaction. The present paper deals with the production of protease by *F. oxysporium* under SSF. Optimization of various culture parameters to enhance the enzyme production has been investigated.

MATERIALS AND METHODS

1. Isolation of Fusarium oxysporum

F. oxysporum isolated from infected soybean seed was maintained on PDA slants at 15°C *F. oxysporum* was identified by using the key of Leslie and Summerell [4].

2. Fermentation

Czapek-dox medium was used with Casein hydrolysate as nitrogen source. 75 ml of medium in Erlenmeyer flask (250 ml) was sterilized at 15 lbs for 20 min. Solid substrate - Groundnut cake, Soybean cake, and Cottonseed Cake were used as carbon source for the production. 20 g of powder cake was taken in Erlenmeyer flask (500 ml) and moistened with mineral salt solution. The substrate was sterilized at 15 lbs for 20 min. Both the Submerged fermentation and Solid-state fermentation media were inoculated with 2 ml of conidial suspension made from 8 days old sporulating PDA slant. Submerged fermentation was carried out for four days at 27°C on orbital shaker at 150 rpm. The flasks were incubated at 27°C for 10 days. The Submerged fermentation medium was filtered through Whatman no 40 filter paper and centrifuged at 2000g for 30 min. The filtrate was dialyzed against distilled water for 24 hr. Thus, filtrate obtained was used as source of proteases for the assay.

3. Purification of protease

Phosphate buffer (200 ml; pH 6.5; 0.1 M) was added to the Solid-state fermented medium and kept on orbital shaker at 200 rpm for 1 hr. The content was filtered with two-fold cheesecloth. The extract was centrifuged and filtered through Whatman no 40 filter paper. The filtrate was dialyzed for 24 hr. against phosphate buffer (pH 6.5). Further

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purification was done by precipitating with chilled acetone and allowed to settle in a refrigerator overnight. The precipitate was separated in a centrifuge at 20000g for 15 min. at 4°C. The powder was dissolved in water and used further. Thus obtained filtrate was used as source of protease. Experiments were carried in triplicate and results are presented as the mean of all the three reading.

4. Assay of protease

Protease was assayed by Kunitz's method [5]. Culture filtrate (1 ml) was mixed with 1 ml of casein substrate (1% w/v) prepared in phosphate buffer (pH 6.9; 0.1M) and digested with TCA (5% w/v) and incubated for 20 min at 45°C. The contents of the test tubes were filtered, added 2 ml of 0.5 N NaOH to 1 ml of filtrate and color reaction was carried out with 0.6 ml of Folin Ciocaltue Reagent (FCR). The optical density was measured at 660 nm and compared with standard curve of tyrosine. One unit of enzyme was defined as the amount of enzyme required to liberate one microgram of tyrosine in 30 min. at 27 °C.

RESULTS AND DISCUSSION

Various solid substrates alone or in combination were screened for higher protease production. However Soybean cake was chosen as best solid substrate and further experiments were carried out using this substrate. Soybean cake (10-50 g) was fermented in separate Erlenmeyer flasks (500 ml) and was tested for high enzyme production. Effect of incubation period (2-10 days) on protease production was studied. The medium was adjusted to range pH (3-7) and subsequent production of enzyme was studied. The effect of temperature to optimize the enzyme production was also studied.

Table 1: Screening of various solid substrates on protease production by *Fusarium oxysporium*

Solid substrate	protease activity (Unit /g substrate)
CC	1.02
GC	1.03
SC	1.16
GC +CC	0.67
CC + SC	0.78
GC + SC	0.82
GC +CC + SC	0.55

(**GC**- Groundnut cake; **CC**- Cottonseed Cake; **SC** -Soybean cake)

So far species of *Aspergillus*, *Penillium*, *Rhizopus* and *Mucor* were used for the production fungal protease, this is the first times Fusarium is employed for the production on solid-state fermentation, as *Fusarium solani* is known to produce protease in plant infection. Generally wheat bran has been extensively used as substrate for the SSF. However only few records are made for the use of Soybean flour [6] with *Penicllium citrinum*, Soybean meal [7] with *Bacillus amyloliquefaciens* and bean cake [8] with *A.niger* for SSF. Among and various solid substrate screened for protease yield on Soybean cake gave maximum enzyme closely followed by Groundnut cake and Cottonseed cake (Table 1).

Table 2: Effect of incubation period on protease production by *Fusarium oxysporium* on Soybean cake

Day	Protease activity (Unit / g substrate)
2	0.05
3	0.08
4	0.14
5	0.28
6	0.49
7	0.74
8	1.19
9	0.95
10	0.82

Protease production by *Fusarium oxysporium* was greater at 30 g of solid substrate as compared to that of high or low substrate levels (Table 3). Eight days of incubation period was optimal to yield high enzyme titres (Table 2). The yield of enzyme in the range 27- 40 °C was significant whereas it was inconsistent in the rest of the range (Table 4).

Table 3: Influence of Substrate concentration on production of protease by *Fusarium oxysporium* on Soybean cake

Substrate concentration (g)	Protease activity (Unit / g substrate)
10	0.63
20	0.95
30	1.20
40	1.16
50	0.98

The optimum pH for the production for the protease was 5.0 to 6.5 (table 5). Fungal acid

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proteases have an optimal pH between 4 and 4.5 and are stable between pH 2.5 and 6.0. Rao et al.[9] had described their usefulness in the cheese making industry due to their narrow pH and temperature specificities

Table 4: Effect of temperature on protease production by *Fusarium oxysporium* on Soybean cake

Temperature °C	**protease activity (Unit /g substrate)**
20	0.21
22	0.45
25	0.78
27	0.92
30	1.12
32	1.19
35	0.97
37	0.90
40	0.87
42	0.58
45	0.31
47	0.11

Table 5: Effect of pH on protease production by *Fusarium oxysporium* on Soybean cake

pH	**protease activity (Unit /g substrate)**
3.0	0.65
3.5	0.67
4.0	0.68
4.5	0.78
5.0	0.81
5.5	0.97
6.0	0.98
6.5	0.88
7.0	0.81

The cost of enzyme production is a major obstacle in the successful application of proteases in industry. Protease yields have been improved by screening for hyperproducing strains and/or by optimization of the fermentation medium. The present investigation could be scaled up to higher levels of protease production using Soybean cake as solid substrate. Characterization and purification of protease is in progress.

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